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SYNOPSIS

"Neutralization of Neuroinflammation by sPLA$_2$-IIA Inhibitors from Wild Edible Fruits"

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INTRODUCTION

Inflammation is a dynamic process that is elicited in response to mechanical injuries, burns, microbial infections and other noxious stimuli that threaten the well-being of the host. Inflammation is a normal and prerequisite of healing of the injured tissue, However, it's a double-edged sword, with undesirable consequences such as systemic shock and circulatory collapse, if prolonged leads to local tissue damage in many organs. The inflammatory response can destroy healthy tissue and cause more damage than the original problem, as a result, uncontrolled inflammatory reactions are the biochemical basis for the origin of a chronic inflammatory disease like rheumatoid arthritis, coronary heart disease, diabetes or asthma and cancers, figure- 1(1-4). Hence inflammatory diseases contribute too much clinical suffering and provide major targets for improved treatment.

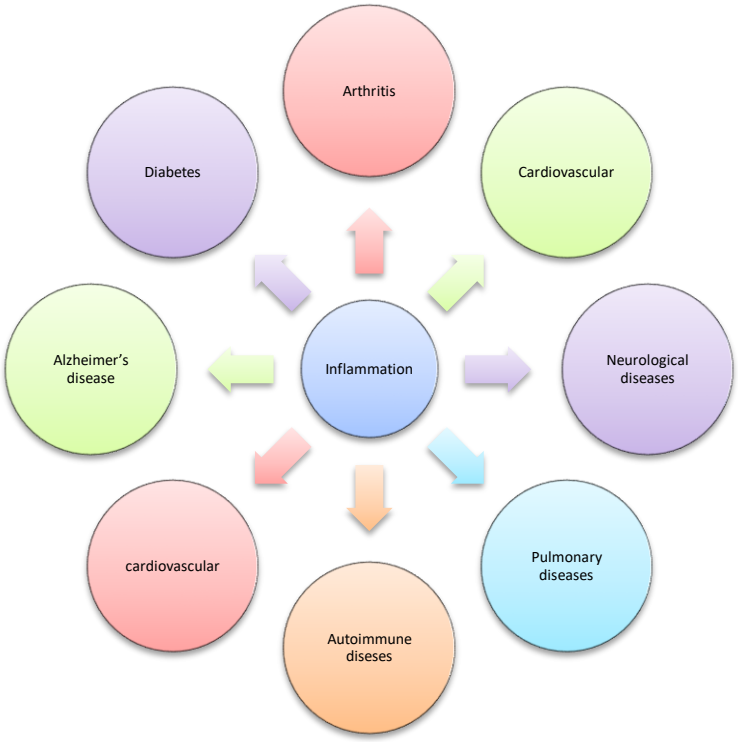
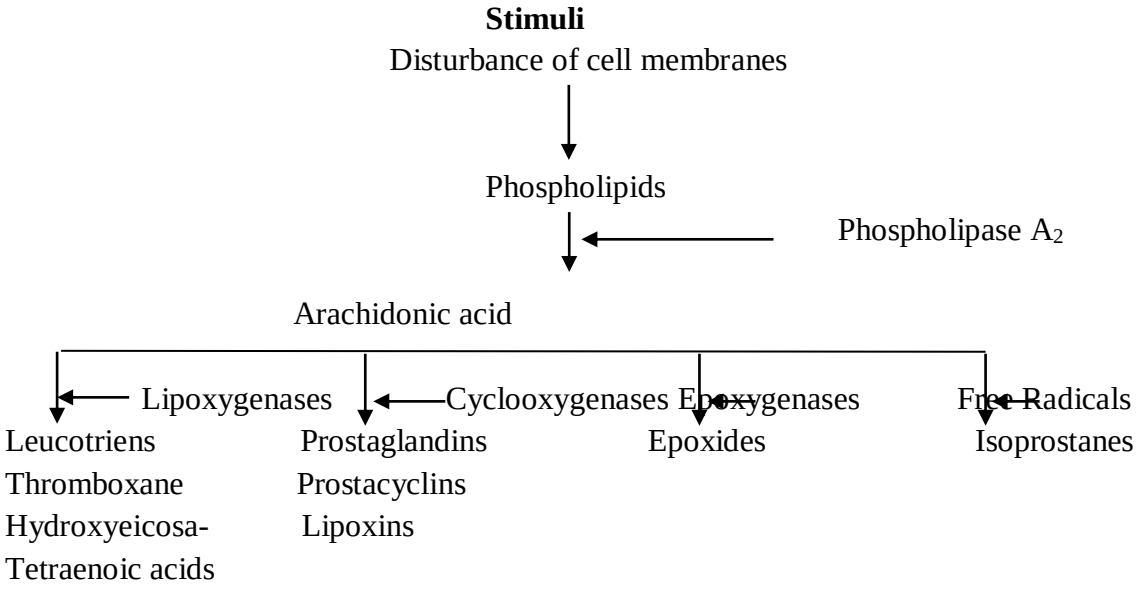


Figure 1 : Inflammtion contributes to many diesases

Inflammation mainly mediated by secretory phospholipases A_2 ($sPLA_2s$) (EC 3.1.1.4.) and are known to regulate the arachidonic acid pathway by which pro-inflammatory mediators of inflammation are released. In our study, we are precisely focusing on secretory phospholipase A_2 enzyme, which is commonly involved in arachidonic acid cascade plays a key role in inflammatory responses and chronic inflammatory diseases such as arthritis, gouts, neurodegenerative diseases, bowel inflammatory diseases, different cancers(5), gastrointestinal disorders etc.



Schematic representation of Synthesis of eicosanoids from arachidonic acid

To treat such inflammatory diseases there are anti-inflammatory therapies including, non-steroidal anti-inflammatory drugs that inhibit COX1/2 enzyme, thus preventing the conversion of arachidonic acid to PGH2, but currently, the NSAIDs drugs are most widely used in the treatment of acute inflammatory disorders, despite their renal, gastric negative secondary effects. (6). Now there is a need for a safe, potent, nontoxic or a lesser toxic anti-inflammatory drug.

$sPLA_2$ IIA enzymes are elevated in neuroinflammatory diseases such as ischemia and Alzheimer's disease (62-63). The $sPLA_2$ IIA activate cytosolic phospholipase A2 ($cPLA_2$), which catalyze arachidonic acid synthesis. Cyclooxygenase-2(COX-2) catalyzes the biosynthesis of prostaglandin E_2 (PGE_2) from arachidonic acid (64). Excess influx of calcium activates $sPLA_2$ IIA which mediates initiation of apoptosis, activation of Protein Kinase C (PKC), cytosolic PLA_2 and mitogen activated protein kinase (MAPK). Intracellular calcium also linked to production of reactive oxygen species through activation of NADPH oxidase, which is involved in the neurotoxicity of Alzheimer's disease. NADPH oxidases catalyze the production of reactive oxygen species which in turn it can stimulate ERK1/2 phosphorylation that in turn activate of $cPLA_2$ (65).

MAP Kinases such as ERK1/2 and p38 are major regulators of proinflammatory cytokines such as PGE_2, TNF-α, IL-6 and IL-1ß. The $cPLA_2\alpha$ mediate the biosynthesis of PGE_2 (59), the prominent marker of neuroinflammation in many CNS and peripheral neurological disorders. Several studies demonstrated that MAPK activate $cPLA_2\alpha$ that results the release of lipids, from which the synthesis proinflammatory cytokines (66, 67). However, it is not clear

whether ERK1/2 or p38 kinase plays a key role in the deleterious effect on LPS infusion in animal model. Activated astrocytes and microglia are the main sources of PGs during neuroinflammation (68, 69). The astrocytes are important immunocompetent cells to cause neuroinflammatory diseases such as Parkinson's disease (70) and stroke (71). The astrocytes are also important sensor of invading pathogens including bacteria and viruses (72). The synthesis and release of proinflammatory mediators, i.e., chemokines and eicosanoids in astrocytes can be induced by bacterial endotoxins (73).

The recent experimental evidence showed that the notion of $sPLA_2$ IIA are causally implicated in systemic pathobiology of neurodegenerative disorders, thus mandate the need of potent specific PLA_2 inhibitors as preventive and/or therapeutic agents. There are several inhibitors are identified for $sPLA_2$ IIA and their isoforms as anti-inflammatory molecules from different sources such as natural sources (74-77) and synthetic drugs (78-81) amid continuous search for potent PLA_2 inhibitor. Surprisingly, there is less evident of $sPLA_2$ IIA inhibitors for neuroinflammation therapy and is merelyneglected such an important piece of research work. This thought enlightens us to explore natural products, i.e. phytochemicals as well as crude extracts from wild edible fruits for inhibition of $sPLA_2$ IIA enzymes of neuroinflammatory disorders.

Recent studies showed that bacterial endotoxin, LPS (lipopolysaccharide) exposure mediate time and dose dependent increase of $sPLA_2$-IIA mRNA expression in murine primary astrocytes (82). Therefore, murine primary astrocytes culture will be used to study the role of $sPLA_2$ IIA in LPS induced *in vitro* model. The *in vivo* model of neuroinflammation would be developed by the intracerebroventricle microinjection of LPS to explore the regulation of MAPK-cPLA_2a-PGE_2 pathways in mice cerebral cortex which would justify clinical testing in brain diseases to which neuroinflammation contributes. Further, the LPS induced *in vitro* and *in vivo* neuroinflammation models are subjected to neutralization by inhibition of $sPLA_2$ IIA with treating extracts of wild edible fruits and their bioactives.

Wild edible plants (WEPs) mean species that are neither cultivated nor domesticated, but are available from their wild, and used as sources of food and medicines. From the past, edible wild fruits have played a very vital part in supplementing the diet of the people. Edible wild fruits play significant role in the rural economy of the many districts of India providing nutrient food supplement and also by generating side income to the poor people (83). The cattle grazers, wood cutter, poachers and forest trekkers generally use these fruits in the forests (84). The

dependence on these fruits has gradually decline as more exotic fruits have been introduced. Apart from their traditional use of food, potentially they have many advantages. They are edible and having nutritional food value, which provides the minerals like sodium, potassium, magnesium, iron, calcium, phosphorus etc. They are immune to many diseases and often used in different formulation of 'Ayurveda' in Indian Folk- medicine.

Some botanical exploration and publications have emphasized on the diversity and food value of wild edible fruit plants (85, 86, 87,88,89, 90, 91). Fruits are rich source of iron, high amount of pectin and contain vitamin c. Food value shows proteins (0.4%), carbohydrates (0.5-0.9%), sugars (7-11%), fibers (0.5-1%) and ascorbic acids. The fruits are very sour at maturity but sourish sweet when ripe. It can be eaten raw or for making pickles, jam, jelly, squash, syrup and chutney. It is used for toxicity, bronchitis and to cure anemia. Ethno botanically the fruits are used as an astringent, antiscorbutic, and as a remedy for biliousness. Wild edible plants promote good health by assisting in preventing cancer and high blood pressure, stimulating the immune system improving drug metabolism and tissue regeneration (92-93). However, there is no concrete report about the pharmaceutical potential of wild edible fruits from the study area. Hence the study is undertaken.

The present context, the plant medicines are of great importance in primitive healthcare. According to the World Health Organization (WHO), about 80% of the world population relies mainly on plant and fruits-based drugs. In the indigenous systems of medicines of Ayurveda, Siddha, Chinese folk medicines and Unani, a large number of wild edible fruits were used for the treatment of human diseases (7). Hence in our studies, we have selected some of wild edible fruits to validate and they were subjected to *in-vitro* assays like antioxidant activity, anti-inflammatory activity, $sPLA_2$ inhibition and in vivo such as edema, hemolysis assay.

OBJECTIVES

1. Screening of potent anti neuroinflammatory wild edible fruits.
2. Neutralization of neuroinflammatory activity of wild edible fruit extracts- *invitro* and *in vivo* studies.
3. Estimation of neuroinflammatory markers in vitro and in vivo models.

METHODOLOGY

Materials

Secretory phospholipase A_2 IIA, Enzyme Linked Immuno Sorbent Assay kit were purchased from the Cayman chemicals, Ann Arbor, USA. Cell culture plates from Biolite - Thermo. BSA Sephadex G-25, Sephadex G-50 and Sephadex G-75, CM-Sephadex C-25, and LPS (L2018) were purchased from Sigma-Aldrich. Cell culture medium DMEM, FBS and D-PBS were from HiMedia, India. EDTA and DMSO were from Sisco Research Laboratories, India. All chemicals used in this study were standard laboratory grade.

Fruit collection

Wild edible fruits were collected from, Kodagu, Hassan and Mysore regions of Western Ghats, India. The fruits were identified with the help of Dr. Thoyajaksha, Associate Professor of Botany, Maharani's Science College for Women, Mysore.

Sample preparation

The fresh wild edible fruit samples were collected and washed to remove surface microbes or pollutants. The samples were shade dried. The fruit samples were ground to fine powder using a motorized grinder. To prevent the degradation of bioactive substances the powdered samples were stored in an airtight container at 4°C temperature until used.

Extraction of wild edible fruits

75g of powder of wild edible fruits was packed in Whatman No. 1 paper, and then extracted using solvents such as hexane, benzene, chloroform, acetone, ethanol, methanol and water with the Soxhlet apparatus. The powder to solvent ratio was 75 grams to 500 millilitres (weight to volume). The extracts were reduced to powder by lyophilization process. The yield was calculated as a percentage of weight to weight of crude sample.

Phytochemical analysis

***Test for alkaloids*:** Wagner's reagent was used for alkaloid analysis in fruit sample. Fruit sample was added to Wagner's reagent. Alkaloids were confirmed when brown or reddish precipitate was produced.

***Test for flavonoids*:** Concentrated sulphuric acid was added to fruit extract. The developing orange colour confirms flavonoids.

***Test for terpenoids*:** 2 ml of fruit extract, 2ml of chloroform and 3 ml of conc. sulphuric acid were mixed. Terpenoids were confirmed by the developing a reddish layer at the contact.

***Test for steroids*:** To 2 ml of fruit extract, 2 ml of chloroform and 3 ml of conc. sulphuric acid were added. Steroids were confirmed by forming an upper red layer and lower acid layer.

***Test for glycosides*:** Liebermann's test; fruit extract was added to acetic acid and chloroform (2 ml each). After cooling, conc. H_2SO_4 was added. The presence of glycosides was indicated by the appearing green colour.

***Test for tannins and phenols*:** Ferric chloride test; fruit extract was added to 0.3% ferric chloride, presence of tannins and phenols were indicated by a brownish-green or black- blue colouration**.**

***Test for saponins*:** Foam Test; Fruit extract was diluted to 5 ml of water; the presence of saponins was indicated by forming foam**.**

Purification of sPLA$_2$-IIA

The method of Kasturi and Gowda (1989), was used to extract the sPLA$_2$-IIA from the venom of *V. russellii* was purified to homogeneity. In brief, 0.05 M phosphate buffer pH 7.0 was used to fractionate 110 mg of *V. russellii* venom on a Sephadex G-75 column (1.5 x 110 cm). Three fractions were extracted from the venom. sPLA$_2$-IIA activity was detected in the second fraction, which makes up around 30% of the total protein loaded. Following lyophilization, this sPLA$_2$-IIA fraction was handed over to CM Sephadex-C-25 column (2.5 x 20 cm) chromatography. Phosphate buffers with different molarities and pH values (0.05 M to 0.2 M, pH 7.0 to pH 8.0) were used to elute the fractions in a stepwise manner. They were divided into two portions, II and V, respectively. The two eluted fractions above resemble the protein profiles of II and V of Kasturi and Gowda (1989). The lyophilised fractions II and V were then eluted using 0.05 M phosphate buffer pH 7.0 and subjected to Sephadex G-50 column (1.0 X 40 cm) chromatography. Both peaks displayed sPLA$_2$-IIA activity and were uniform and SDS-PAGE (Laemmli, 1970) was used to verify homogeneity.

Molecular docking

Protein target preparation

Molecular docking is a computational procedure used to predict the interaction between a small molecule (ligand) and a protein target. In this study, we aim to achieve molecular docking of bioactive molecules (inhibitors) with secretory phospholipase A2 group IIA (sPLA$_2$-IIA)

enzyme, specifically using AutoDock Vina. In the protein preparation step obtain the 3D structure of the $sPLA_2$-IIA enzyme from the Protein Data Bank (PDB) using PDB ID: 1POE. Remove water molecules, cofactors and hetero atoms attached to 1POE using PyMOL. Add the hydrogen atoms and assign the charges to the protein using AutoDockTools (ADT). Create the PDBQT file, containing both the atomic coordinates and the charges.

Ligand preparation

The phytocompounds were retrieved from PubChem database in 2D form and optimized them using molecular mechanics to ensure proper geometry of the ligands by using the MMFF94 force field. Added the hydrogen atoms and assigned the partial charges for the ligands, modified the optimized ligand structures to PDBQT format using AutoDockTools (ADT).

Molecular docking simulation study

The binding pocket of $sPLA_2$-IIA was determined by analyzing the enzyme's known binding sites in the literature, grid box size setting is adjusted to encompass the binding site residues. The standard docking protocol using AutoDock Vina is implemented to create the Grid Parameter File (GPF) and then generate the grid maps. AutoDock Vina was executed using the graphical interface tool PyRx.

Antioxidant activity

DPPH method (2, 2- diphenyl1-picrylhyorazyl) for radical scavenging assay

Using the Blois method, an *in vitro* antioxidant activity was examined for wild edible fruits extracts. DPPH working solution of 0.1 mM concentration was mixed with 0.5 ml of the fruit extract and allowed for radical scavenging at room temperature for 30 minutes in the dark room. The absorbance was measured at 517 nm. The antioxidant activity of fruit extracts was calculated using the following formula.

$$\%\ \text{Antioxidant activity} = \frac{Absorbance\ of control - Abosrbance\ of sample}{Absorbance of control} \times 100$$

Total phosphomolybdenum assay

The phosphomolybdenum reduction assay was carried out to determine the overall antioxidant capability of fruit extracts. The fruit extracts of 10, 15, 20, 25, and 30 µg/ml were combined with 1.5 ml of phosphomolybdenum reagent, which consisted of 0.6M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. Then the mixture was incubated at 95°C for 90 minutes. The absorbance was measured at 695 nm.

Lipid peroxidation assay

Lipid peroxidation was induced by adding $FeCl_3$ (7 mM) to a solution containing extracts/inhibitors and mice liver homogenate (10%). The reaction was terminated by adding 2 ml of ice-cold HCl containing thiobarbituric acid (TBA) (0.38%) and TCA (4%) mixture after 30 min of incubation. The mixture was vortexed thoroughly and incubated for one hour in a boiling water bath. Lipid hydroperoxides released were extracted with butanol and measured at 530 nm (Halliwell & Gutteridge, 1989).

sPLA$_2$-IIA assay (Colorimetric method)

The activity of phospholipase A_2-IIA was evaluated using a novel colorimetric technique derived from the free fatty acid measurement method established by Nov-x (1965). For fruit extracts, the reaction commenced with the addition of 0.01 ml (25 µg) of phospholipase A_2 to a mixture including 1000 nmoles of egg phosphatidylcholine, 0.3 ml of ether, 0.5 ml of 0.05 M Tris-HCl buffer at pH 7.5, and 40 nmoles of Ca^2. The tube contents were spun for 1 minute and centrifuged at 500 x g for 5 minutes at room temperature. The total lipids were extracted into the upper organic phase. An 800µl upper organic phase was transferred into 800µl of chloroform: petroleum ether (5:1, v/v) and 0.5 ml of freshly produced cobalt reagent in a test tube. The reagent includes 1.35 ml triethanolamine, 6 g $Co(NO_3)_2$ +6 H_2O +0.8 ml glacial acetic acid, 100 ml saturated K_2SO_4 solution, and 7 ml saturated Na_2SO_4 solution. After mixing for 1 minute, the tube was centrifuged for 10 minutes at 1200 g at room temperature. Cobalt selectively makes soap along with fatty acids recovered from the higher organic phase. This phase (0.6 ml) was carefully transferred to a tube with 0.75 ml of indicator solution (0.4% 1-nitroso-2-naphthol in 96% ethanol). The amount of cobalt present directly affects the intensity of the coloured reaction with 1-nitroso-2-naphthol. After 30 minutes, the solution was diluted to 3.35 ml with 2 ml of 96% ethanol and measured at 500 nm using a spectrophotometer. Linoleic acid was used as standard fatty acid.

Effect of calcium and substrate concentration on sPLA$_2$-IIA inhibition

To study the mode of sPLA$_2$-IIA inhibition, calcium concentration from 100 to 500 µM and substrate concentration from 5 to 30 nM of diheptanoyl Thio- PC on sPLA$_2$-IIA inhibition was examined. The experiments were carried out independently with IC_{50} concentrations of inhibitors. The enzyme's activity was assumed to be 100%, and inhibition was calculated as a percentage of the residual activity.

Intrinsic fluorescence interaction study

Horiba Jobin Yvon Fluorolog-3 spectrofluorimeter was used to evaluate the relative intrinsic fluorescence intensity of $sPLA_2$-IIA with and without inhibitors at the Centre of excellence and Nanoscience (CeNS, Bangalore, India). The various concentration of (0.02 to 0.1 µM) of inhibitor was mixed with Tris HCl buffer (0.1 M; pH 7.4), calcium (5 mM) and $sPLA_2$-IIA (20µg/ml) enzyme. After the excitation at 280 nm, fluorescence spectra were recorded between 300 and 370 nm. The standard (tryptophan) was used to correct non-specific quenching of spectra generated internal absorption and filtration by inhibitors.

Circular dichroism study

Spectra of Far UV-CD were recorded for $sPLA_2$-IIA enzyme (30µg/ml) with or without inhibitor (IC_{50} concentration) in standard reaction mixture using Jasco J-810 spectropolarimeter (Centre of excellence and Nanoscience (CeNS), Bangalore, India). The spectra were obtained between 200 and 250 nm at room temperature. The response time was 2s and the bandwidth was 1 nm. A total of ten scans were used to get the final spectrum. The spectrum of the blank solution containing Tris-HCl buffer (100 mM, pH 7.4) and 5 mM calcium was subtracted to correct the protein spectra by using K2D3 software.

Neutralization of indirect hemolytic activity

Boman and Kaletta (1957) method was used to estimate $sPLA_2$-IIA induced indirect hemolytic activity. The substrate was prepared by suspending 1 ml of packed fresh human RBC and 1 ml of egg yolk in 8 ml of PBS. The extracts/inhibitor with $sPLA_2$-IIA (30µg) was pre-incubated at 37ºC for 30 minutes. Then 1 ml of the substrate was added and allowed to react for 45 minutes at 37ºC. The reaction was terminated by the addition of 9 ml of ice-cold PBS. The reaction mixture was centrifuged for 20 minutes at 1,500 g. The hemolytic activity of enzyme was determined in terms of hemoglobin released, which was read at 530 nm. The $sPLA_2$-IIA enzyme with extract/inhibitor was used as a positive control reaction mixture.

Neutralization of edema inducing activity

Yamakawa and Hokama (1976) procedure and modified by Vishwanath and Gowda (1987) was used to investigate the neutralization of edema-inducing activity of $sPLA_2$-IIA activity. Initially, $sPLA_2$-IIA (5µg) enzyme was injected into the intra plantar surface of the right hind footpad of mice (20-25µg) either alone or combined with different concentrations of extracts/inhibitors in a total volume of 20µl saline. Control was received 20µl of saline or vehicle. After 45 minutes, anesthesia (pentobarbitone, 30 mg/kg, i. p.,) was given to mice and sacrificed. The lower two

limbs were surgically amputated at the ankle joint and weighed separately. The percentage increase in weight attributable to edema was calculated by the ratio of the weight of the edematous limb to the weight of a standard (sham-injected) limb × 100.

Cell culture

Newborn Wistar rat pups, aged zero to one day, were obtained from the Experimental Animals Centre (EAC) at NGSM, Nitte. Primary astrocyte cultures were derived from the cortices of Wistar rat pups aged 0-1 day, utilizing a modified method based on previously described techniques (Haseleu et al., 2013). Cortical tissue was excised, and the meninges were extracted in Petri dishes with D-Hanks balanced salt solution. The tissue was meticulously chopped using scalpels, subsequently suspended in a 0.25% trypsin solution (10:1 volume ratio) and incubated at standard condition. An equivalent volume of high-glucose DMEM containing 10% FBS was added to terminate trypsinization. Following centrifugation (5 minutes at 200g, 25°C), and the cell pellet was resuspended in high-glucose DMEM containing 5.5 mM glucose, 20% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin..

Production of neuroinflammation model

Male C57BL/6 mice, aged 6 to 8 weeks, were acquired and housed in polypropylene cages at 21°C, subjected to a reverse 12-hour light/dark cycle, with unrestricted access to water and normal rodent diet. All experimental protocols were approved by the Institutional Animal Care and Use Committee NGSM Nitte, and complied with the US National Institutes of Health guidelines for laboratory animal care.

Intracerebroventricular (ICV) microinjections were conducted in accordance with established protocols (Paxinos & Watson 1986), with minor changes. Lipopolysaccharide (LPS) was prepared at a concentration of 1 mg/ml in sterile saline solution. On testing day, mice was sedated via intraperitoneal injection of ketamine of concentration 100 mg/kg and xylazine, 10 mg/kg concentration, thereafter positioned in a stereotaxic apparatus. Following the shaving and sterilization of the scalp, a tiny aperture was created at 2.0 mm lateral to the midline and 1 mm posterior to the bregma. Mice were either an intracerebroventricular injection of 2.5 mg of LPS or an intracerebroventricular injection of extract one hour before LPS delivery, utilizing a Harvard apparatus syringe pump. The control group mice were administered an intracerebroventricular injection of either 2.5 µl of saline or 2 µl of DMSO.

Western blotting

SDS-PAGE gel apparatus was assembled, and the tank was filled with 1X running buffer. Equal amounts of protein was loaded (usually 20-30 µg) into each well. The ladder was loaded in one lane would serve as molecular weight marker. After that the gel was ran at 80-100 volts through the stacking gel, then increased to 120-150 volts in the resolving gel until bands were well-separated. Membrane was wetted in methanol (for PVDF) and then equilibrated in transfer buffer. The membrane "sandwich" was assembled (gel, membrane, and filter papers) according to the manufacturer's instructions, removing air bubbles. The proteins were transferred from the gel to the membrane. (Wet transfer: ran at 100 V for 1-2 hours at 4°C. Semi-dry transfer: Typically took 15-30 minutes at 15-25 volts).The membrane was blocked with 5% non-fat dry milk or 5% BSA in TBST for 1 hour at room temperature or overnight at 4°C with gentle rocking. BSA was used instead of milk if detecting phosphorylated proteins. Primaryantibody was diluted in blocking buffer or TBST. The membrane was incubated with the primary antibody for 1-2 hours at room temperature or overnight at 4°C with gentle rocking. The membrane was washed with TBST three times for 5-10 minutes to remove unbound antibody**.**

Estimation of pro inflammatory mediators by ELISA

All reagents, samples, and standards are allowed to reach room temperature before conducting the assay (approximately 20-30 minutes). Standards were prepared in different concentrations ranging from 0.01 ng/ml to 10 ng/ml as per the instructions. Samples were diluted to required concentrations of standard curve range. Standard curve wells (duplicate or triplicate for each concentration), Blank wells (containing only assay buffer), and Sample wells (test samples in duplicate or triplicate). Pipetted 50-100 µl of each standard into the designated wells, and 50-100 µl of sample was added into the respective wells.

Estimation of prostaglandin E_2

The levels of PGE_2 were quantified according to the manufacturer's methodology. 50 µl of standard diluent was added to the wells designated as blanks; conversely, 50 µl of serially diluted standards and samples were added to the corresponding wells. Subsequently, introduced biotinylated PGE_2 antibody to each well, sealed the plate, and incubated at 37°C for one hour. Upon completion of the incubation, removed the seal and disposed of the samples; thereafter, rinsed the wells with diluted wash buffer. Introduce 100µl of Streptavidin-HRP conjugate, mixed thoroughly, sealed, and incubated the plate at 37°C for one hour.

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